Almond witches'-broom phytoplasma: a potential threat to almond, peach, and nectarine

Yusuf Abou-Jawdah, Hala Dakhil, Shoaa El-Mehtar, and Ing-Ming Lee

Abstract: A lethal phytoplasma disease of almond, almond witches'-broom (AlmWB), spread rapidly in Lebanon, killing about a hundred thousand trees within 10 years. This phytoplasma was the first member of the pigeon pea group reported to infect stone fruits. Preliminary results of grafting experiments proved that AlmWB could be transmitted by grafting to almond, peach, and nectarine but not to apricot, plum, and cherry. The occurrence of this disease at altitudes ranging from sea level to about 1000 m makes it a major potential threat to almond, nectarine, and peach in the major stone fruit production areas. Using universal primers, nested polymerase chain reaction is normally required for detection; however, the new primers described here allow efficient detection from the first run of polymerase chain reaction.

Key words: phytoplasma, almond witche's-broom, almond, stone fruits, *Prunus* spp., PCR detection.

Résumé : Le balai de sorcière de l'amandier (AlmWB), une maladie létale de l'amandier causée par un phytoplasme, s'est rapidement répandue au Liban, tuant une centaine de milliers d'arbres en 10 ans. Ce phytoplasme a été le premier membre du groupe du pois cajan retrouvé dans des fruits à noyaux. Les résultats préliminaires d'expériences de greffage ont montré que l'AlmWB pouvait être transmis par la greffe à l'amandier, au pêcher et au nectarinier, mais pas à l'abricotier, au prunier et au cerisier. La présence de cette maladie à des altitudes allant du niveau de la mer jusqu'à environ 1000 m en fait une menace majeure pour la production d'amandes, de nectarines et de pêches dans les principales régions productrices de fruits à noyaux. Avec des amorces universelles, il est habituellement nécessaire d'utiliser la technique d'amplification en chaîne par polymérase nichée dans des tests de détection, alors que les nouvelles amorces que nous décrivons permettent une détection dès la première amplification en chaîne par polymérase.

Mots clés : phytoplasme, balai de sorcière de l'amandier, amandier, fruits à noyaux, *Prunus* spp., détection, amplification en chaîne par polymérase.

Introduction

An epidemic of almond witches'-broom (AlmWB) caused devastating losses in almond production in Lebanon during the last decade. The disease spread rapidly, killing about a hundred thousand trees in 10 years (Abou-Jawdah et al. 2002). Symptoms of infection include witches'-brooms arising mainly from the main trunk and roots, early flowering, stunted growth, leaf rosetting, dieback, off-season growth, and proliferation of slender shoots. The causal organism was identified as a phytoplasma closely related to, but distinct from, members of the pigeon pea witches'-broom phytoplasma group (16SrIX) (Abou-Jawdah et al. 2002).

Stone fruits are affected by several diseases associated with plant pathogenic phytoplasmas. In Europe, European stone fruit yellows (ESFY) phytoplasma (Ahrens et al. 1993; Lorenz et al. 1994) was reported to cause significant losses in yield of apricot (Morvan 1977), plum (Guinchedi et al. 1982), cherry (Bernhard et al. 1977), and peach (Poggi Pollini et al. 1993). This phytoplasma belongs to the 16SrX group (apple proliferation group). Other important phytoplasmas of stone fruits include: X-diseases of peach and choke cherry (16SrIII group), and peach yellow leaf roll (PYLR; 16SrX group) in North America; apricot chlorotic leaf roll (ACLR; 16SrX group) from Italy; and cherry lethal yellows (CLY; 16SrV group) from China (Blomquist and Kirkpatrick 2002; Lee et al. 1998).

A preliminary survey indicated that the primary host of AlmWB is almond, since the areas infested were mainly almond-growing areas with a limited number of other stone-fruit species present. To assess the potential economic

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Y. Abou-Jawdah, H. Dakhil, and S. El-Mehtar. Faculty of Agricultural and Food Sciences, American University of Beirut, P.O. Box 11-0236, Beirut, Lebanon.
I.-M. Lee.[1] Molecular Plant Pathology Laboratory, Plant Sciences Institute, U.S. Department of Agriculture, Beltsville, MD 20705, U.S.A.

[1]Corresponding author (e-mail: leeim@ba.ars.usda.gov).

impact of this newly described pathogen, it is important to study its host range. In this note, we report that almond witches'-broom phytoplasma may pose a potential threat not only to almond but also to peach and nectarine. We also report an improved primer set useful for the detection of AlmWB phytoplasma in infected almond, peach, and nectarine.

Materials and methods

Grafting experiments

Two-year-old seedlings of almond, apricot, cherry, nectarine, peach, and plum (Table 1) were purchased from a local nursery in the Bekaa region where AlmWB disease is unnoticeable. Thirty seedlings randomly selected from the nursery tested negative for AlmWB and other phytoplasmas by nested polymerase chain reaction (PCR). In mid-June 2001, scions (grafting buds) were collected from almond trees and nectarine seedlings naturally infected with AlmWB phytoplasma that were previously diagnosed by nested PCR as described below. Nine seedlings from each species were grafted and three remained as controls. Each seedling was T-grafted with three grafting buds. Of the nine grafted seedlings, six received grafting buds originating from almond and three from nectarine.

Data on disease symptoms were recorded during late winter and early spring 2002. The presence of AlmWB phytoplasma in symptomatic tissues above the scion was confirmed by nested PCR. In November, December, and then starting from March till August, all seedlings were tested by nested PCR, using universal primers at monthly intervals to assess infection.

Nucleic acid extraction and PCR amplification

The techniques were previously described (Abou-Jawdah et al. 2002). Leaf midribs were chilled with liquid nitrogen and pulverized. A buffer, containing 2% cetyltrimethylammonium bromide (CTAB), 1.4 M NaCl, 20 mM ethylenediaminetetraacetic acid (EDTA), 1% polyvinylpyrrolidone (PVP), 0.2% mercaptoethanol, 100 mM Tris–HCL, pH 8.0, was added to the crushed tissue (800 mL/100 mg), mixed by frequent vortexing, and incubated at 60°C for 20 min. Then, 600 µL of chloroform–isoamylalcohol (24:1) was added. Following vortexing and centrifugation at 14 000 × *g*, the supernatant was mixed with an equal volume of ice-cold isopropanol and incubated at –20°C for 20 min. After centrifugation for 8 min at 14 000 × *g*, the pellet was washed with 80% ethanol, air dried, suspended in 50 µL sterile water, and maintained at –20°C until use.

In nested PCR, the universal primer pair P1/P7 (Schneider et al. 1995), which primes a fragment approximately 1800 base pairs (bp) that extends from the 5′ end of the 16S rDNA to the 5′ region of the 23S rDNA, was used in the first run. Then nested PCR was performed with primers R16F2n/R16R2 (Gundersen and Lee 1996) that amplifies a 1200-bp fragment. Amplifications were performed with a thermocycler, Icycler (Bio-Rad, Richmond, Calif.) in 20-µL reactions containing 200 mM each of the four deoxyribonucleotide triphosphates (dNTPs), 0.5 µM of each primer, 2 mM $MgCl_2$, 1× polymerase buffer, 1 unit *Taq* (Abgene Surrey, U.K.), and 1- to 2-µL sample DNA. Two PCR protocols were followed, one for the universal primers and one for the semispecific primers as described below. The PCR reactions, using the universal primers, consisted of 1 cycle at 95°C for 2 min, 35 cycles at 94°C for 30 s, 50°C for 30 s, and 72°C for 2 min, and a final extension step at 72°C for 7 min. A total of 1.6 µL of diluted (1:40) PCR product from the first amplification was used as template for nested PCR. When the semispecific primers ALW-F2/ALW-R2 were used, two modifications were performed: an annealing temperature of 44°C for 30 s was used and the extension time was reduced to 30 s. The amplified products were analyzed by electrophoresis in 1.5 or 2% agarose gel, followed by staining with ethidium bromide.

A semispecific primer pair was designed based on the 16S rDNA sequence of AlmWB, using BLAST (Basic local alignment search tool) (Altschul et al. 1990) analysis to confirm specificity: ALW-F2 5′-AGAGTAGCTACAACG TGAGTT-3′ and ALW-R2 5′-GAGCTATAGGCCCAGGAT-3′. The primers amplify a 390-bp fragment of the 16S rDNA.

To confirm the specificity of the new primers, simultaneous runs of nested PCR with the universal primers listed above, or with the new primers (a single PCR amplification), were conducted with samples that contained different phytoplasmas representing nine groups: tomato big bud (16SrI), peanut witches'-broom (16SrII), X-disease phytoplasma (16SrIII), elm yellows phytoplasma EY1 (16SrV), clover proliferation (16SrVI), ash yellows (16SrVII), pigeon pea witches'-broom (16SrIX), apple proliferation phytoplasma (16SrX), and Mexican periwinkle virescence (16SrXIII).

RFLP analysis of nested-PCR-amplified 16S rDNA

Nested-PCR products amplified using primer pair P1/P7 followed by primer pair R16F2n/R16R2 were analyzed by single restriction endonuclease digestion, selecting one enzyme (HpaII) that gives a similar pattern for samples of the pigeon pea group and three enzymes (HaeIII, RsaI, AluI) that distinguish AlmWB from closely related phytoplasmas (Abou-Jawdah et al. 2002). The restriction products were then separated by electrophoresis through a 2% agarose gel and stained in ethidium bromide. DNA bands were visualized with a UV transilluminator.

Results and discussion

The grafting experiments showed clearly that AlmWB can be graft transmitted to almond, nectarine, and peach (Table 2). Symptoms were clearly seen starting in February 2002. Both sources of scions (almond and nectarine) gave similar transmission efficiency. The most apparent symptom on almond, nectarine, and peach was the development of bushy growth at the base of the stem (rootstock) at or below the soil level. The stems were succulent, with shortened internodes, and the leaves were small and light green in color (Fig. 1). New growth above the scion, in almond, peach, and nectarine, was considerably weaker than in their respective controls; internodes were shorter, leaves were lighter green and smaller and showed high susceptibility to infection by powdery mildew (Fig. 2). The presence of AlmWB phytoplasma in the symptomatic samples was confirmed starting in early March by nested PCR, using univer-

Table 1. Rootstocks and scions of two-year-old *Prunus* spp. seedlings used in the graft transmission assay of AlmWB phytoplasma.

Scion	Rootstock	Propagation	Origin of rootstock
P. armeniaca (apricot), undefined	Undefined (8 apricots and 1 peach)	Seedling	Lebanon
P. salicina (Japanese plum) 'Friar' and 'Blackamber'	Myrobalan (plum)	Seedling	Vilmorin, Paris, France
P. persica (peach) 'Royal Glory'	Montclar (peach)	Seedling	Vilmorin, Paris, France
P. avium (sweet cherry), local brown cherry	*Prunus mahaleb*	Seedling	Lebanon
P. amygdalus (almond), local firik	Wild local almond	Seedling	Lebanon
P. persica (nectarine) 'Jad'	Montclar	Seedling	Vilmorin, Paris, France

Table 2. Development of symptoms in six stone-fruit species following grafting with AlmWB-infected grafting buds.

	Major symptoms					
Botanic species	Small leaves	Short internodes	Witches'-broom	No. symptomatic/ no. grafted	Susceptibility to powdery mildew	Presence of AlmWB phytoplasma
Almond	++	++	++	9/9	++	+
Nectarine	++	++	++	9/9	++	+
Peach	++	++	++	9/9	++	+
Apricot	–	–	–	1/9[a]	–	–
Plum	–	–	–	0/9	–	–
Cherry	–	–	–	0/9	–	–
Control	–	–	–	0/9	–	–

Note: Grafting was done on 10 June 2001; grafting buds were grafted near the base of the scion. Clear symptoms were first recorded in February. Nested PCR was performed to detect the presence of AlmWB at monthly interval from November 2001 till August 2002. Positive PCR results were obtained from symptomatic tissues (++) above the scion as early as March 2002; negative results were recorded during the testing period from March to August 2002.

[a]Witches'- broom symptoms were observed on one rootstock (the peach rootstock), but not the other eight apricot rootstocks.

Fig. 1. Healthy and AlmWB-infected seedlings of nectarine. following grafting with infected scions. Note the proliferation at the base of the infected seedling and the size of leaves as compared with the control on the left.

Fig. 2. Healthy (left) and infected peach seedling (right) after graft transmission of AlmWB phytoplasma. Note the smaller size and chlorotic leaves of the infected seedling and its susceptibility to powdery mildew.

sal primers (Fig. 3). The presence of phytoplasma was detected after the first PCR run, using primers P1/P7 with extracts from witches'-broom tissues below the scion (data not shown). However, nested PCR with universal primers (P1/P7 followed by R16F2n/R16R2) were required to detect phytoplasma in extracts from parts above the scions, indicating a higher phytoplasma concentration in the tissues with witches'-broom. Restriction fragment length polymorphism (RFLP) analysis of the nested-PCR products further confirmed the identity of AlmWB in the three hosts (Fig. 4).

On apricot and plum, even though the grafting buds were still viable, no symptoms were observed on the parts above

Fig. 3. Gel electrophoresis of nested-PCR-amplified products in 1.5% agarose. A and H, 200-bp ladder; B, D, and F, extracts respectively from symptomatic almond, peach, and nectarine grafted with AlmWB-infected scions; C, E, and G, their respective controls. Nested PCR was performed using primers P1/P7 followed by R16F2n/R16R2.

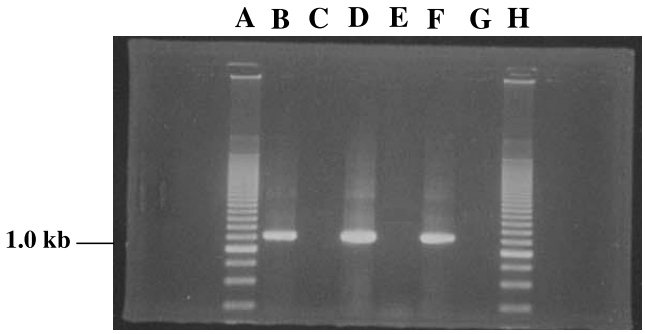
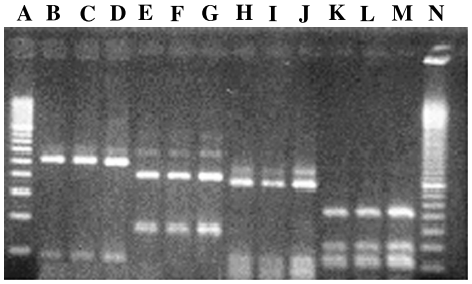


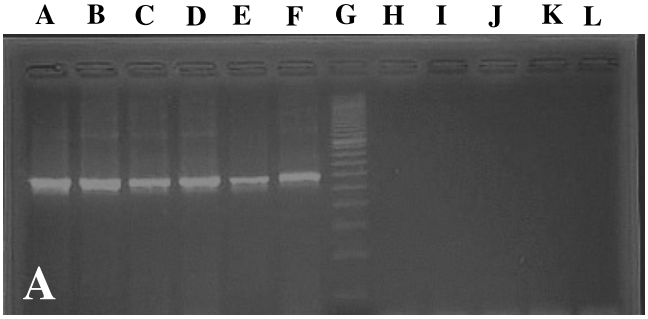
Fig. 4. RFLP analyses of phytoplasma 16S rDNA sequences (nested-PCR products amplified using primer pair R16F2n/R16R2) from the graft-infected almond, nectarine, and peach, respectively. DNA products were digested with HaeIII (B, C, and D), HpaII (E, F, and G), AluI (H, I, and J), or RsaI (K, L, and M). A and N represent 200- and 100-bp ladders, respectively. Digested products were electrophoresed through a 2% agarose gel and stained in ethidium bromide.

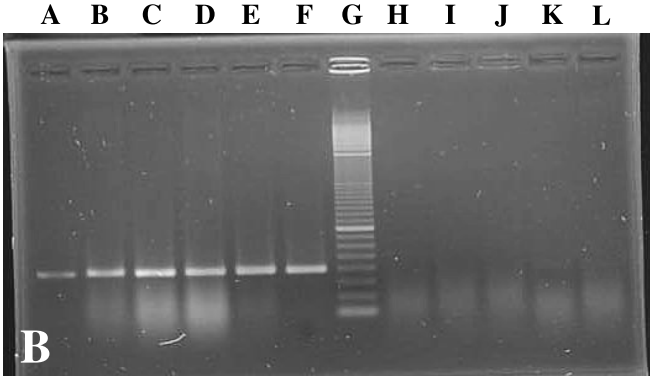


the grafting buds and growth was similar to the controls. The absence of AlmWB phytoplasma in these symptomless tissues was confirmed by nested-PCR analysis conducted at monthly intervals till the end of August 2002. The results showed that the new growth was not infected 14 months after the graft inoculation. We noted that in one apricot seedling, which was not grafted on apricot but on the peach rootstock, witches'-broom symptoms appeared on the rootstock but not on apricot, indicating that apricot may not be totally immune. However, since a long incubation period for phytoplasma diseases has been reported, such observations must be interpreted with caution. Pastore et al. (2001) reported that symptoms appeared 1.5 to 2 years following grafting of ESFY-infected grafting buds onto apricot and plum.

On cherry, the almond and nectarine grafting buds were all dead, the growth was delayed until early May, and no differences were observed from the control. Analysis by nested PCR gave negative results. These preliminary results indicate that apricot, plum, or cherrie, depending on altitude, may serve as replacement crops in AlmWB-infested areas, until resistant almond cultivars become available. On the other hand, AlmWB poses a great threat to almond, nectarine, and peach production because it seems to be effectively transmitted by an as yet unidentified vector. Our preliminary data has indicated that a few nectarine trees were infected by AlmWB phytoplasma (Abou-Jawdah et al. 2002). Our survey showed that in Lebanon, the most frequently found leafhopper on stone fruits is *Assymetrasca decedens* (Paoli). Preliminary transmission tests in insect-proof cages, using this leafhopper were not successful, indicating that an unknown vector may be present in low population densities or may live on other hosts and use stone fruits as a transient host.

Fig. 5. (A) Results of nested PCR using the primer pair P1/P7 followed by R16F2n/R16R2. (B) Single amplification PCR using the semispecific primers ALW-F2/ALW-R2. PCR products were electrophoresed through a 2% agarose gel and stained in ethidium bromide. Samples A–F are from infected almond trees, whereas samples H–L are from healthy trees; G, 200- and 100-bp ladders in gels for (A) and (B), respectively.





Differences in susceptibility to infection by ESFY of various rootstocks and varieties were reported (Kison and Seemüller 2001). All stone fruits in Europe are affected by severe-decline diseases associated with ESFY (Jarausch et al. 1998). Lee et al. (1995) reported mixed infections of stone fruits by phytoplasmas; the introduction of AlmWB may also lead to mixed infections that would further aggravate the situation.

For diagnostic purposes, rapid but sensitive and reliable tests are required. Nested PCR is costly in terms of time and reagents, whereas the development of a direct PCR (single amplification) method reduces costs and testing time. The extracts of 20 samples, in which phytoplasma could only be detected after nested PCR, using the universal primers, were tested with the designed semispecific primer pair. The results showed clear bands of about 390 bp after

one PCR run with primer pair ALW-F2/ALW-R2 (Fig. 5). Of nine standard samples tested, representing diverse phytoplasma groups, only pigeon pea witches'-broom gave a positive reaction, indicating that these primers may be specific to the pigeon pea group alone. All nine phytoplasma samples were positive in nested PCR with universal primers (data not shown).

Stone fruits are among the major fruit crops grown in the world, including the U.S.A. (California), South-European countries, and central Asia. Our study has shown that the lethal AlmWB disease presents a potential threat to peach and nectarine in Lebanon.

Even though wild almonds are native to Lebanon and stone-fruit cultivation started over 100 years ago, AlmWB was introduced only recently. Therefore, stricter certification and quarantine measures on the movement of stone-fruit germplasm should be imposed. The rapid rise in the importance of phytoplasma diseases makes it imperative to standardize diagnostic techniques that can be used routinely in certification of stone fruits for the detection of phytoplasmas.

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